

SITEK RESEARCH LABORATORIES

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FINAL REPORT

Study Title

In Vivo Test for Chemical Induction of Micronucleated
Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Test Article

Dimethylamine-2-2ethyl azide (DMAZ)

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Laboratory Project I.D.

SITEK Study No. 0976-1521

Study Initiation Date

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Sponsor

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14. ABSTRACT Dimethylamine-2-2ethyl azide (DMAZ, 99.3% pure) was tested for its potentials to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and female CD-1 mice according to OECD TG 474 in compliance with Good Laboratory Practice. Male and female mice were dosed orally at 125, 250, and 500 mg/kg. Animals were euthanized approximately 24 and 48 hrs after dosing. The percentage of polychromatic erythrocytes (PCE) and the frequency of micronucleated polychromatic erythrocyte (MPCE) were determined at 24 and 48 hrs. Cytotoxicity was assessed by scoring the number of PCE and norchromatic erythrocyte (NCE) in first 200 erythrocytes for each animal. There were no statistically significant increases in the number of MPCE in the treated groups when compared to the concurrent vehicle control group. These results indicate that DMAZ did not cause chromosome damage in the in vivo mouse micronucleus assay and therefore it is not considered to be a clastogenic agent.					
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STUDY DIRECTOR'S COMPLIANCE STATEMENT

Study No.: 0976-1521

Sponsor's Test Article I.D.: Dimethylamine-2-2ethyl azide (DMAZ)

The study described in this report was conducted in compliance with the following test guidelines:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Section 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002, (1).

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997, (2).

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996, (3).

The study described in this report was conducted in compliance with the following Good Laboratory Practice standard, except as indicated in the last paragraph:

United States Food and Drug Administration Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2006, (4).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005, (5).


Japanese Ministry of Agriculture, Forestry and Fisheries, 11 Nohsan, Notification No. 6283, October 1, 1999, (6).

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997, (7).

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984 (8).

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998, (9).

The strength and stability of the test article, dosing solutions and controls, under the experimental conditions, were not determined.

Signature:  Date: 6-25-08
Jian Song, Ph.D.
Study Director

QUALITY ASSURANCE UNIT'S STATEMENTStudy No. 0976-1521Sponsor's Test Article I.D.: Dimethylamine-2-2ethyl azide (DMAZ)

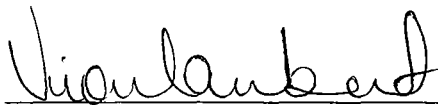
The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 01/15/08

The following phases were inspected for this study:

<u>Inspection Date</u>	<u>Phases Inspected</u>	<u>Date Findings Reported to Study Director</u>	<u>Date Findings Reported to Management</u>
<u>03/14/08</u>	<u>Randomization of the animals</u>	<u>03/14/08</u>	<u>03/31/08</u>
<u>04/28/08</u>	<u>Workbook Audit</u>	<u>04/29/08</u>	<u>04/30/08</u>
<u>05/1/08</u>	<u>Draft Report</u>	<u>05/1/08</u>	<u>05/02/08</u>
<u>06/23/08</u>	<u>Final Report</u>	<u>06/25/08</u>	<u>06/25/08</u>

Signature: _____

Vian Lambert, B.S.
Quality Assurance Manager

Date: _____

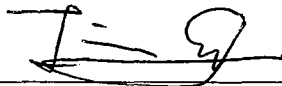
06/25/08

STUDY DIRECTOR'S SIGNATURE PAGE

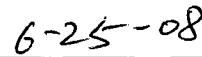
This study was performed under the supervision of Shambhu K. Roy and Jian Song, Ph.D. Study Directors for In Vivo Micronucleus Studies, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report on this study was written by Dr. Jian Song and Karen S.K. Shore. It was released by Dr. Song on June 25, 2008.

Signature

A handwritten signature in black ink, appearing to be 'Jian Song', written over a horizontal line.

Jian Song, Ph.D.
Study Director

A handwritten date '6-25-08' in black ink, written over a horizontal line.

Date

ABSTRACT

The test article, dimethylamine-2-ethyl azide (DMAZ, 99.3% pure), was tested for its potential to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and female CD-1 mice. Water was used as the vehicle control. The test article and vehicle control were administered by oral gavage. Three male and three female mice per dose group were used in the Range Finding Test. Five male and five female mice per dose group per harvest time were used in the Micronucleus Assay.

The Range Finding Test was performed at 10, 50, 100, 500, 1000 and 2000 mg/kg. All animals, male and female in the 2000 mg/kg dose group, as well as one male and one female in the 1000 mg/kg dose group were found dead on Day 1. All females and two males in the 500 mg/kg dose group exhibited a hunched back on Day 1. All females and males in the 500 mg/kg dose group also exhibited a marked decrease in activity when compared to the vehicle control group on Day 1. On Day 2, two females and two males in the 100 mg/kg dose group exhibited a marked decrease in activity when compared to the vehicle control group. No abnormal clinical signs were observed in any of the surviving animals on Days 3 and 4.

Test article doses of 125, 250 and 500 mg/kg for the Micronucleus Assay were selected based on the results of the Range Finding Test. The highest dose was selected to exclude lethality in accordance with U.S. E.P.A., OECD and ICH guidance documents. The positive control group received a single, oral gavage dose of cyclophosphamide (CP) at 80 mg/kg body weight. Animals were euthanized approximately 24 and 48 hours after dose administration. Positive controls were included in the 24-hour harvest only.

In the Micronucleus Assay, the percentage of polychromatic erythrocytes (PCE) and frequency of micronucleated polychromatic erythrocytes (MPCE) were determined at approximately 24 and 48 hours after the dose administration. Two thousand PCE per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCE and normochromatic erythrocytes (NCE) in the first 200 erythrocytes for each animal. There were no statistically significant increases in the number of MPCE in the treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups or SITEK's historical vehicle control data. Reduction in the percentage of PCE was used as an indication of toxicity. No reduction (more than 20% versus that of the vehicle control) in the percentage of PCE was observed at any dose level or harvest time.

The frequency of MPCE in each treatment group was compared to that in the respective vehicle control using a one-tailed Student's T-test. The vehicle and positive control groups met the criteria for a valid test. The data from all the dose groups treated with DMAZ, at both bone marrow harvest time points, were not significant when compared to

the vehicle control group. Cyclophosphamide (CP), the positive control, showed a statistically significant increase in MPCE when compared to the concurrent vehicle control, thus fulfilling the criteria for a valid assay. All remaining criteria for a valid assay were also met. In the vehicle control, the average number of MPCE per 2000 PCE did not exceed 10. Five animals from each sex were alive at the time of euthanasia for each dose level. Therefore, the results of this assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, the test article was negative in the Micronucleus Assay. It was concluded that the test article did not cause chromosome damage *in vivo* and, therefore is not considered to be a clastogenic agent.

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INTRODUCTION

This study was conducted by Shambhu K. Roy, Ph.D., Jian Song, Ph.D., Hussain Shaffi, B.S., Weiyu Xie, M.D., Karen S. K. Shore, B.A., Adrienne Parker, B.S. and Ann Nguyen, A.A., at SITEK Research Laboratories from March 4, 2008 to April 20, 2008. The experimental procedures used to perform this study were essentially those of J.A. Heddle (10), W. Schmid (11), K.H. Mavournin, et al (12), M. Hayashi, et al (13) and R.R. Tice and M.D. Shelby (14).

The purpose of this study was to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes (MPCE) in mouse bone marrow cells (10, 11). Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (12-14).

MATERIALS

TEST ARTICLE

- | | |
|--------------------------|--|
| 1. Name: | Dimethylamine-2-ethyl azide (DMAZ) |
| 2. CAS No.: | 86147-04-8 |
| 3. Provided by: | MACH I, Inc., 340 East Church Road, King of Prussia, PA 19406, USA |
| 4. Batch/Lot No.: | 3M DMAZ L-15686, Lot 8 |
| 5. Physical Description: | Clear liquid |
| 6. Shipping Conditions: | Room Temperature |
| 7. Date Received: | January 10, 2008 |
| 8. Storage Conditions: | Refrigerated (1-5 °C) |
| 9. Purity: | 99.3% |
| 10. Expiration Date: | N/A |

A Certificate of Analysis for the test article is presented in Appendix IV.

CONTROL SUBSTANCES

Positive Control

Cyclophosphamide (CP), which induces micronucleus formation, was used at 80 mg/kg body weight (8.0 mg/mL x 10 mL/kg) by oral gavage in this study. The information on the CP used in this study is provided below:

- | | |
|------------------------|-----------------------|
| 1. Source: | Sigma (St. Louis, MO) |
| 2. CAS No.: | 6055-19-2 |
| 3. Lot No.: | 075K1661 |
| 4. Storage Conditions: | Refrigerated (1-5°C) |
| 5. Purity: | 99.7% by HPLC |
| 6. Expiration Date: | May 2, 2011 |

Sterile water (Baxter, Lot No. C715987) was used to dissolve the positive control. The expiration for this lot was July 2008.

Vehicle Control

Water was used as the vehicle control.

The information on the water used in this study is provided below:

1. Source:	Baxter
2. CAS No.:	7732-18-2
3. Lot No.:	C715987
4. Storage Conditions:	Refrigerated (1-5°C)
5. Expiration Date:	July 2008

TEST ANIMALS

Approximately 42-day-old, male and female, CD-1 mice were obtained from Harlan Sprague Dawley, Inc. (Frederick, MD).

The animals were housed three or five animals per cage during the study. Hardwood chip bedding, free of injurious substances, was used. The animals received Purina Certified Rodent Diet (Code No. 5002C, Lot No. Nov 18 07 3C and Lot No. DEC 30 07 1A, Brentwood, MO) and fresh tap water *ad libitum*. Levels of contaminants present in the food and water were within acceptable levels. Bedding was changed at least twice per week.

The animals were quarantined for at least 7 days for the Micronucleus Assay prior to dose administration. The animals were observed each day and the observations, temperature and humidity of the animal room were recorded in the study notebook. Throughout the trials, the animal room was maintained at 17-23°C and 17-54% humidity. A 12-hour diurnal light cycle was employed.

A total of 42 mice (21 males, 21 females) were used for the Range Finding Test. A total of 90 mice (45 males, 45 females) were used for the Micronucleus Assay.

EXPERIMENTAL PROCEDURE

DOCUMENTATION

Detailed documentation of the procedures, results and methods used for the analysis of the results of this study were entered into the study notebook. The study notebook also includes the original protocol, study report copies and all relevant communications with the Sponsor.

TEST SYSTEM IDENTIFICATION

All of the animals to be dosed received an ear tag with a number unique to the particular study. All of the cages were assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director and the animal study proposal number (06-02-12) approved by SITEK's IACUC. The microslides were labeled with SITEK's study number, the last three digits of the animal number as a code number and the date of slide preparation.

SOLUBILITY TEST

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test was performed.

The test article was tested for its solubility/miscibility in deionized, distilled water. A nonviscous liquid will be tested for miscibility in weight per volume. The solubility/miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test would consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article was dissolved or until 1.5 mL of vehicle had been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in aliquots of 0.5 mL until 5.0 mL had been added. The volume of vehicle required for complete dissolution, and any additional observations, would be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle would be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test would be conducted. 1.5 mL of vehicle in 0.1 mL increments would be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in 0.5 mL increments until 5.0 mL had been added. The resulting solution would be thoroughly mixed and observed for miscibility. The test article would be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations would be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article would be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data were available, the solubility/miscibility test would not be performed.

RANDOMIZATION OF TEST ANIMALS

Upon arrival, the animals were randomly assigned to clean, polycarbonate cages. All of the animals were ear tagged with a number unique to the study. In the Range Finding Test, the animals were assigned to experimental groups (three animals per sex), without regard to body weight, prior to dose administration.

In the Micronucleus Assay, the animals were weighed and placed into weight groups. Each weight group spanned 1 gram. Using a computer-generated random matrix, the animals then were assigned sequentially from the weight groups to randomized cages corresponding to the treatment groups (five animals per sex per group).

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

The stock solutions for the Range Finding Test and the Micronucleus Assay were prepared as specified in the dilution scheme which was kept in the study notebook. The specified amount of test article was weighed and the required volume of water was added to reach the highest stock concentration. The remaining concentrations were made by subsequent dilution.

The dosing solutions were prepared by SITEK study personnel just prior to treatment. In all treatments, the amount of vehicle administered to the animals was limited to a level which would have no significant toxic effect. The strength and stability of the test article and the test article dosing solutions under the experimental conditions was not determined by SITEK Research Laboratories.

TREATMENT PROCEDURE

The animals were treated with a single dose by oral gavage. The positive control (CP) was also given as a single dose by oral gavage. The total dose volume administered to the animals was 10 mL/kg body weight for each test article dose level and vehicle control. 10 mL/kg body weight was also used for the positive control.

RANGE FINDING TEST

In the Range Finding Test, treatment groups of three mice per sex were treated at six test article dose levels of 10, 50, 100, 500, 1000 and 2000 mg/kg body weight and one group was treated with the vehicle control.

The animals were observed for 3 days following dose administration for treatment-related clinical signs and/or death. Body weights were checked on the day of dosing (Day 1) and at the end of the 3-day observation period (Day 4). At the end of the 3-day observation period, the animals were euthanized. The body weight data were entered into the computer to calculate the mean body weight and percent change in the mean body weight, using a MS Office Excel 2003 spreadsheet program. Clinical signs were entered into the computer in tabular form to demonstrate frequency. Doses for the Micronucleus Assay were selected based on the results of the Range Finding Test.

MICRONUCLEUS ASSAY

The Micronucleus Assay was performed at 125, 250 and 500 mg/kg body weight based on the results of the Range Finding Test.

The animals were randomized and placed into treatment groups of five mice per sex. Two such treatment groups were designated for each of the test article dose levels and the vehicle control per harvest (a total of 80 mice). One group was designated for the positive control (10 mice). Water and CP were used as the vehicle control and positive control, respectively. The dose volume was 10 mL/kg body weight for the test article, vehicle control and positive control.

The mice were euthanized by CO₂ asphyxiation approximately 24 or 48 hours after the dose administration. Five male and five female mice were euthanized from each test article dose level and the vehicle control at each harvest time. Five male and five female mice treated with the positive control were euthanized 24 hours after treatment.

After each animal was euthanized, the groin area was cleansed with 70% ethanol, and the femurs were exposed by cutting into the skin and muscle of the thighs. The femurs were separated just above the kneecaps, and the heads of both femurs were removed with scissors. The bone marrow from the femurs was flushed into a disposable culture tube, containing 1.0 mL of fetal bovine serum, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes were centrifuged at 800 rpm for 5 minutes. The supernatant was removed, leaving approximately 0.1 mL of serum above the cell pellet. The cells were resuspended by flicking the tube until a homogeneous suspension was observed.

A small drop of the cell suspension was placed on the unfrosted end of a clean microslide and spread along the length of the slide. The slides were allowed to air dry, then fixed in methanol for 15 minutes and allowed to air dry again. The slides were stained in Wright-Giemsa stain for 2-4 minutes, then rinsed in distilled water, allowed to air dry completely, and mounted in Cytoseal using #1 cover glasses. The backs of the slides were cleaned with methanol.

The slides were scored "blind" in order to avoid bias on the part of the scorers. First, the number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) among 200 erythrocytes (PCE + NCE) per animal was determined. The number of micronucleated polychromatic erythrocytes (MPCE) then was determined for 2000 PCE per animal (3).

STATISTICAL ANALYSIS

The data from the score sheets were consolidated into summary sheets and entered into a computer, using an MS Office Excel 2003 spreadsheet program. The dose group means were calculated for the percentage of PCE as well as the frequency of MPCE. A significant reduction in percent PCE (more than 20% versus that of the vehicle control) was used as an indication of toxicity.

Data were analyzed separately for male and female animals. The frequency of MPCE in each dose group was compared to that in the respective vehicle control group using a one-tailed Student's t-test (10). An Excel 2003 statistical package was used to calculate p values for the t-test. The results were considered significant if the p value was ≤ 0.025 . Statistical analysis was not performed on values that were lower than or equal to those of the respective vehicle control. The Cochran-Armitage Test (trend test) (11) was used to analyze trends if the Student's t-test showed a positive result. The trend test was considered significant if the p value was ≤ 0.05 . For this study no trend test was needed.

CRITERIA FOR A VALID ASSAY

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed 10.
2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.
3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

EVALUATION OF TEST RESULTS

Positive Response

The test article was considered to have caused a positive response in this assay if:

1. The test article showed a positive dose-response trend and a statistically significant increase ($P \leq 0.025$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article caused a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may have been compared to historical vehicle control data. For this study the data from 48-hour harvest in female mice have been compared to historical vehicle control data (see Table 14)
2. In the event there was no positive dose-response trend, at least two consecutive test doses must have produced a statistically significant increase in the number of MPCE.

Negative Response

The test article was considered to have caused a negative response if none of the test doses showed a statistically significant increase in the number of MPCE when compared to the vehicle control.

Equivocal Response

The test article was considered to have caused an equivocal response if the test article induced a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend. In such a case, a repeat assay would have been performed only with the approval of the Sponsor.

ARCHIVES

The raw data, protocol, protocol amendments, protocol deviations, documentation, and a copy of the Final Report, and an electronic file containing the data tables and Final Report of the study, will be maintained for ten years in SITEK Research Laboratories' archives located at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY

A miscibility test was performed. The information specifying 99.3% purity of dimethylamine-2-ethyl azide (DMAZ) was provided by the sponsor. The test article solution was miscible with the vehicle (water) at 200 mg/mL.

RANGE FINDING TEST

The Range Finding Test was performed at 10, 50, 100, 500, 1000 and 2000 mg/kg. The animal body weight range on the day of dose administration was 30-36 grams for the males and 25-32 grams for the females. The changes in the mean body weight for each dose group, which occurred by the end of the 3-day observation period following dosing, are presented in Table 1 (Appendix I). No reduction of greater than 8.8% in the mean body weight was observed in any other dose group of either sex. The mortality of mice following oral administration is presented in Table 10.

All animals, male and female in the 2000 mg/kg dose group, as well as one male and one female in the 1000 mg/kg dose group were found dead on Day 1. All females and two males in the 500 mg/kg dose group exhibited a hunched back on Day 1. All females and males in the 500 mg/kg dose group also exhibited a marked decrease in activity when compared to the vehicle control group on Day 1. On Day 2, two females and two males in the 1000 mg/kg dose group exhibited a marked decrease in activity when compared to the vehicle control group. No abnormal clinical signs were observed in any of the surviving animals on Days 3 and 4. Clinical signs were observed in the 3-day observation period. The data are presented in Tables 2-9 (Appendix I).

MICRONUCLEUS ASSAY

Based on the toxicity results from the Range Finding Test, the doses selected for the Micronucleus Assay were 125, 250 and 500 mg/kg body weight. The animal body weight range on the day of dose administration was 31-36 grams for the males and 23-31 grams for the females.

The percentage of PCE and the MPCE frequency were determined from bone marrow preparations from animals sacrificed approximately 24 and 48 hours after dose administration. Individual results for the dose levels and vehicle groups in the Micronucleus Assay for the animals sacrificed approximately 24 and 48 hours after treatment are presented for the males in Tables 11 and 13, respectively, and summarized in Table 15 and for the females in Tables 12 and 14, respectively, and summarized in Table 16 (Appendix I).

No significant reduction of the percentage of PCEs was observed at any test article dose level or harvest time in either sex. The ranges of the mean numbers of MPCE in 2000 PCE from this assay are summarized below:

<u>Treatment</u>	<u>Mean Numbers of MPCE in 2000 PCE in Males</u>	
	<u>24 Hours</u>	<u>48 Hours</u>
Vehicle Control	0.6	0.4
Test Article Doses (125-500 mg/kg body weight)	0.4-0.8	0.2-0.6
Positive Control-CP (80 mg/kg body weight)	46.8*	N/A

<u>Treatment</u>	<u>Mean Numbers of MPCE in 2000 PCE in Females</u>	
	<u>24 Hours</u>	<u>48 Hours</u>
Vehicle Control	0.6	0.0**
Test Article Doses (125-500 mg/kg body weight)	0.2-0.4	0.0-0.6
Positive Control-CP (80 mg/kg body weight)	31.6*	N/A

*Statistically significant response using a one-tailed Student's t-test

** MPCE in the concurrent vehicle control was 0 which is unusually low. Therefore, the data from female mice harvested at 48 hours was compared to SITEK's historical vehicle control data (Appendix II).

There was no evidence of a significant increase in the number of MPCE in the test article-treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups or SITEK's historical vehicle control data.

CP, the positive control, showed a statistically significant increase in MPCE (Tables 11 and 12). The mean numbers of MPCE in 2000 PCE were 46.8% and 31.6% for the male and female mice, respectively, thus fulfilling the criteria for a valid assay.

All remaining criteria for a valid assay were also met. In the vehicle control, the average number of MPCE per 2000 PCE did not exceed 10. Five animals from each sex were alive at the time of euthanasia for each dose level.

CONCLUSIONS

The test article, dimethylamine-2-2ethyl azide (DMAZ, 99.3% pure), was tested for its potential to induce MPCE in the bone marrow cells of male and female CD-1 mice.

The results of the assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, DMAZ was negative in the Micronucleus Assay at 125, 250 and 500 mg/kg body weight. Therefore, it was concluded that DMAZ did not cause chromosome damage *in vivo* and is not considered to be a clastogenic agent.

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APPENDIX I

DATA TABLES

TABLE 1

RESULTS OF RANGE FINDING TEST

MOUSE BODY WEIGHTS INITIALLY AND APPROXIMATELY 72 HOURS
FOLLOWING ORAL ADMINISTRATION OF DMAZ

STUDY NO.: 0976-1521

VEHICLE: Water (10 mL/kg)

MALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
MEAN BODY WEIGHT	34	30	31	34	34	34	34
	36	33	33	33	35	33	33
	34	32	31	35	32	33	32
	35	32	32	34	34	33	33
MALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
MEAN BODY WEIGHT	34	32	32	33	34	30	**
	36	32	33	34	35	31	**
	35	30	31	35	32	**	**
	35	31	32	34	34	31	**
% CHANGE MEAN BODY WEIGHT	0.0%	-3.1%	0.0%	0.0%	0.0%	-8.8%	**
FEMALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
MEAN BODY WEIGHT	26	27	26	30	27	31	27
	28	32	26	26	27	27	27
	27	26	27	29	29	25	28
	27	28	26	28	28	28	28
FEMALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
MEAN BODY WEIGHT	26	26	24	29	27	**	**
	28	30	25	27	26	26	**
	26	25	26	30	30	21	**
	27	27	25	29	28	24	**
% CHANGE MEAN BODY WEIGHT	0.0%	-3.6%	-3.8%	3.6%	0.0%	-7.7%	**

NOTE: Body weights are in grams. Only surviving animals are used for calculating %Change Mean Body Weight.

* Body Weights were taken prior to dosing.

** Animal found dead prior to 72 hours.

Verified by: QA VL SD JS

TABLE 2

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: MALE

Test Article: DMAZ

DAY: 1

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back					2/3		
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions						1/3	3/3
- Inactivity					3/3		
- Tremors						3/3	3/3
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3			
Death						1/3	3/3

* Vehicle used for this study was water (10mL/kg).

** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 3

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: FEMALE

Test Article: DMAZ

DAY: 1

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back					3/3		
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions						1/3	3/3
- Inactivity					3/3		
- Tremors						3/3	3/3
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3			
Death						1/3	3/3

* Vehicle used for this study was water (10mL/kg).

** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 4

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: MALE

Test Article: DMAZ

DAY: 2

SIGNS	DOSE LEVELS (mg/kg)***						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity						2/2	
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3		
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead Day 1.

*** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 5

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: FEMALE

Test Article: DMAZ

DAY: 2

SIGNS	DOSE LEVELS (mg/kg)****						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity						2/2***	
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3		
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead on Day 1.

*** One animal found dead Day 1.

**** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 6

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: MALE

Test Article: DMAZ

DAY: 3

SIGNS	DOSE LEVELS (mg/kg)****						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	2/2***	
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead on Day 1.

*** One animal found dead Day 1.

**** 3 mice per/sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 7

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: FEMALE

Test Article: DMAZ

DAY: 3

SIGNS	DOSE LEVELS (mg/kg)****						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	2/2***	
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead on Day 1.

*** One animal found dead Day 1.

**** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 8

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: MALE

Test Article: DMAZ

DAY: 4

SIGNS	DOSE LEVELS (mg/kg)****						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	2/2***	
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead on Day 1.

*** One animal found dead Day 1.

**** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

TABLE 9

RESULTS OF RANGE FINDING TEST**CLINICAL SIGNS OBSERVED**

SITEK Study No.: 0976-1521

SEX: FEMALE

Test Article: DMAZ

DAY: 4

SIGNS	DOSE LEVELS (mg/kg)****						
	Vehicle*	10	50	100	500	1000	2000**
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Wet Groin							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	2/2***	
Death							

* Vehicle used for this study was water (10mL/kg).

** All animals found dead on Day 1.

*** One animal found dead Day 1.

**** 3 mice per sex dosed at each dose level.

Verified by: QA VL SD JS

Table 10

RESULTS OF RANGE FINDING TEST**Mortality of Mice Following Oral
Administration of DMAZ**

SITEK Study No.: 0976-1521

Vehicle: Water (10 mL/kg)

DOSE LEVEL	SEX	NUMBER OF DEATHS ON DAY				TOTAL NUMBER OF DEATHS
		1	2	3	4	
Vehicle	M	0	0	0	0	0
Vehicle	F	0	0	0	0	0
10 mg/kg	M	0	0	0	0	0
10 mg/kg	F	0	0	0	0	0
50 mg/kg	M	0	0	0	0	0
50 mg/kg	F	0	0	0	0	0
100 mg/kg	M	0	0	0	0	0
100 mg/kg	F	0	0	0	0	0
500 mg/kg	M	0	0	0	0	0
500 mg/kg	F	0	0	0	0	0
1000 mg/kg	M	1	0	0	0	1
1000 mg/kg	F	1	0	0	0	1
2000 mg/kg	M	3	0	0	0	3
2000 mg/kg	F	3	0	0	0	3

Verified by: QA VL SD JS

TABLE 11
MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE
ORALLY ADMINISTERED DMAZ
24 HOURS AFTER TREATMENT

STUDY NO.: 0976-1521

Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL PCE	COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C2482	120	80	60.0	1	
VEHICLE	C2491	127	73	63.5	1	
VEHICLE	C2510	120	80	60.0	0	
VEHICLE	C2477	110	90	55.0	1	
VEHICLE	C2486	108	92	54.0	0	
	MEAN	117	83	58.5	0.6	
125	C2524	130	70	65.0	0	
125	C2501	120	80	60.0	2	
125	C2517	102	98	51.0	0	
125	C2476	101	99	50.5	1	
125	C2502	106	94	53.0	1	
	MEAN	112	88	55.9	0.8	0.333
250	C2509	101	99	50.5	0	
250	C2520	119	81	59.5	0	
250	C2499	110	90	55.0	0	
250	C2478	117	83	58.5	1	
250	C2512	103	97	51.5	1	
	MEAN	110	90	55.0	0.4	*
500	C2506	139	69	66.8	0	
500	C2494	111	89	55.5	1	
500	C2514	117	83	58.5	1	
500	C2488	129	71	64.5	1	
500	C2481	133	67	66.5	0	
	MEAN	126	76	62.4	0.6	*
CP**	C2498	79	121	39.5	60	
CP**	C2516	127	73	63.5	48	
CP**	C2483	104	96	52.0	32	
CP**	C2522	85	115	42.5	39	
CP**	C2484	80	120	40.0	55	
	MEAN	95	105	47.5	46.8	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Verified by: QA VL SD JS

TABLE 12
MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE
ORALLY ADMINISTERED DMAZ
24 HOURS AFTER TREATMENT

STUDY NO.: 0976-1521

Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C2525	120 80	60.0	0	
VEHICLE	C2513	118 82	59.0	2	
VEHICLE	C2507	118 82	59.0	1	
VEHICLE	C2530	116 84	58.0	0	
VEHICLE	C2535	145 55	72.5	0	
	MEAN	123 77	61.7	0.6	
125	C2519	140 60	70.0	0	
125	C2563	117 83	58.5	0	
125	C2555	130 70	65.0	1	
125	C2544	112 88	56.0	1	
125	C2543	123 77	61.5	0	
	MEAN	124 76	62.2	0.4	*
250	C2521	129 71	64.5	0	
250	C2554	138 62	69.0	0	
250	C2545	120 80	60.0	1	
250	C2564	110 90	55.0	1	
250	C2556	105 95	52.5	0	
	MEAN	120 80	60.2	0.4	*
500	C2553	140 60	70.0	0	
500	C2565	105 95	52.5	1	
500	C2541	115 85	57.5	0	
500	C2557	126 74	63.0	0	
500	C2539	124 76	62.0	0	
	MEAN	122 78	61.0	0.2	*
CP**	C2529	90 110	45.0	25	
CP**	C2561	60 140	30.0	33	
CP**	C2542	49 151	24.5	32	
CP**	C2558	57 143	28.5	46	
CP**	C2537	50 150	25.0	22	
	MEAN	61 139	30.6	31.6	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Verified by: QA VL SD JS

TABLE 13
MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE
ORALLY ADMINISTERED DMAZ
48 HOURS AFTER TREATMENT

STUDY NO.: 0976-1521

Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL PCE	COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE**
VEHICLE	C2489	74	126	37.0	1	
VEHICLE	C2523	72	128	36.0	1	
VEHICLE	C2479	71	129	35.5	0	
VEHICLE	C2505	89	111	44.5	0	
VEHICLE	C2511	56	144	28.0	0	
	MEAN	72	128	36.2	0.4	
125	C2485	43	157	21.5	0	
125	C2518	80	120	40.0	0	
125	C2490	67	133	33.5	1	
125	C2504	56	144	28.0	0	
125	C2495	68	132	34.0	0	
	MEAN	63	137	31.4	0.2	*
250	C2480	94	106	47.0	1	
250	C2515	64	136	32.0	1	
250	C2508	102	98	51.0	1	
250	C2492	100	100	50.0	0	
250	C2500	107	93	53.5	0	
	MEAN	93	107	46.7	0.6	0.290
500	C2493	71	129	35.5	0	
500	C2496	71	129	35.5	1	
500	C2503	75	125	37.5	0	
500	C2497	51	149	25.5	0	
500	C2487	65	135	32.5	1	
	MEAN	67	133	33.3	0.4	*

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Verified by: QA VL SD JS

TABLE 14
MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE
ORALLY ADMINISTERED DMAZ
48 HOURS AFTER TREATMENT

STUDY NO.: 0976-1521

Vehicle: Water (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL PCE	COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C2549	112	88	56.0	0	
VEHICLE	C2531	103	97	51.5	0	
VEHICLE	C2559	100	100	50.0	0	
VEHICLE	C2536	124	76	62.0	0	
VEHICLE	C2528	94	106	47.0	0	
	MEAN	107	93	53.3	0.0	
125	C2538	69	131	34.5	1	0.385**
125	C2527	99	101	49.5	1	
125	C2562	96	104	48.0	0	
125	C2548	79	121	39.5	0	
125	C2534	115	85	57.5	1	
	MEAN	92	108	45.8	0.6	
250	C2550	56	144	28.0	0	0.385**
250	C2532	106	94	53.0	0	
250	C2552	109	91	54.5	1	
250	C2560	75	125	37.5	1	
250	C2546	112	88	56.0	0	
	MEAN	92	108	45.8	0.4	
500	C2526	61	139	30.5	0	
500	C2547	69	131	34.5	0	
500	C2533	61	139	30.5	0	
500	C2540	72	128	36.0	0	
500	C2551	78	122	39.0	0	
	MEAN	68	132	34.1	0.0	*

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** Since the mean value of vehicle control group was zero, these groups were compared to historical data.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Verified by: QA VL SD JS

TABLE 15

SUMMARY OF MICRONUCLEUS ASSAY RESULTS

Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Male Mice
Orally Administered DMAZ

Study No.: 0976-1521

Vehicle: Water (10 mL/kg)

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	117	83	58.5	-	0.6
24	125	112	88	55.9	-4.4 %	0.8
24	250	110	90	55.0	-6.0 %	0.4
24	500	126	76	62.4	6.7 %	0.6
24	CP*	95	105	47.5	-18.8 %	46.8 **
48	Vehicle	72	128	36.2	-	0.4
48	125	63	137	31.4	-13.3 %	0.2
48	250	93	107	46.7	29.0 %	0.6
48	500	67	133	33.3	-8.0 %	0.4

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

Verified by: QA VL SD JS

TABLE 16

SUMMARY OF MICRONUCLEUS ASSAY RESULTS

Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Female Mice
Orally Administered DMAZ

Study No.: 0976-1521

Vehicle: Water (10 mL/kg)

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	123	77	61.7	-	0.6
24	125	124	76	62.2	0.8 %	0.4
24	250	120	80	60.2	-2.4 %	0.4
24	500	122	78	61.0	-1.1 %	0.2
24	CP*	61	139	30.6	-50.4 %	31.6 **
48	Vehicle	107	93	53.3	-	0.0
48	125	92	108	45.8	-14.1 %	0.6
48	250	92	108	45.8	-14.1 %	0.4
48	500	68	132	34.1	-36.0 %	0.0

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

Verified by: QA VL SD JS

APPENDIX II

SITEK's HISTORICAL DATA FOR VEHICLE
AND POSITIVE CONTROLS

**MOUSE MICRONUCLEUS
HISTORICAL VEHICLE CONTROL DATA
MPCE per 2000 PCEs
October 2004 to April 2008**

	MPCE/ 2000 PCE	MPCE/ 2000 PCE
	<u>Males</u>	<u>Females</u>
Mean	0.5	0.5
Std. Dev.	0.41	0.37
Range	0 - 1.0	0 - 1.0
n=*	28	28

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

**MOUSE MICRONUCLEUS
HISTORICAL POSITIVE CONTROL DATA
(CP 80 mg/kg)
MPCE per 2000 PCEs
October 2004 to April 2008**

	MPCE/ 2000 PCE Males	MPCE/ 2000 PCE Females
Mean	40.2	43.2
Std. Dev.	13.38	14.55
n=*	16	16
Range	18.2 - 76.6	19.6 - 71.8

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

APPENDIX III

STUDY PROTOCOL, PROTOCOL AMENDMENT AND DEVIATIONS

**SITEK RESEARCH LABORATORIES****IN VIVO TEST FOR CHEMICAL INDUCTION OF MICRONUCLEATED
POLYCHROMATIC ERYTHROCYTES IN MOUSE BONE MARROW CELLS**

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign Section 10 to approve the protocol.**

PART ONE**1.0 SPONSOR**Name: USA RDECOM, AMSRD-MSF1.2 Address: Environmental Acquisition & Logistics Sustaining Program
Aberdeen Proving Ground, MD 210101.3 Sponsor's Study Coordinators: Gunda Reddy, Ph.D., DABT**2.0 TESTING FACILITY**2.1 Name: SITEK Research Laboratories2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 208502.3 Study Director: Shambhu K. Roy, Ph.D.**3.0 STUDY NUMBERS*** 3.1 Testing Facility's Study No.: 0976-15213.2 Sponsor's Study No.: N/A**4.0 TEST ARTICLE**

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

* To be completed by the Testing Facility.


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4.1 Identification

 Name: DMAZ (Dimethylamine-2-ethyl azide (CAS Registry No. 86147-04-8))

 Batch/Lot No.: 8
4.2 Description

 Color: Clear

 Physical Form: Liquid
4.3 Analysis

 Purity Information: Not provided

Does the Sponsor require the use of a correction factor to account for impurity?

☐ Yes ☒ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

☐ Room Temperature ☒ Refrigerated (1-5°C)

☐ Frozen (-10 to -20°C)

☐ Other (please specify): _____

 Expiration Date: provided by sponsor

SKR entered
le JS 4-28-08

4.5 Preferred Vehicle (check one):
☒ H₂O ☐ Saline ☐ Corn Oil ☐ DMSO ☐ Ethanol(50% v:v
in H₂O)

☐ Other (please specify): _____

☐ To be decided by the Testing Facility



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6.0 REGULATORY AGENCY SUBMISSION

6.1 Test Design Specifications

This study protocol is designed to meet or exceed the U.S. EPA, ICH and OECD Guidelines specified in the following documents (1, 2, 3):

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

6.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45[ENV/MC/CHEM (98)17], Paris 1998.

Will this study be submitted to a regulatory agency?

 X Yes

 No

If so, which agency(ies)? Worldwide



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7.0 DOSING SOLUTIONS

The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; EPA FIFRA = 40 CFR, 160.113, and OECD GLPs, Section 6.2.

Does the Sponsor want dosing solution analysis?

____ Yes** X No

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and/or stability of the dosing solutions. Stability will only be determined if the dosing solutions are not prepared immediately prior to each use. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and/or stability of the dosing solutions.

Dosing solution analysis will be performed by:

____ SITEK Research Laboratories ____ Sponsor***

What dosing solutions will be analyzed?

From the Range Finding Test?

____ Yes ____ No

From the Assay?

____ Yes ____ No

Which concentration(s)? _____

** Additional charges will apply. See Special Services price schedule.

***Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:

- All subcontract and/or Sponsor Quality Assurance audit findings and comments.

- Any deviations and/or amendments, if applicable.

- An original or copy of the analysis report.

- Location (address) of where the raw data from the analysis will be archived by the Sponsor or Subcontractor.

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.


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What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ Room Temperature _____ Frozen (-10 to -20 °C)

_____ Refrigerated (1-5 °C)

At what temperature should the dosing solutions be shipped?

_____ Room Temperature _____ On Wet Ice

_____ On Dry Ice

8.0 BLOOD (PLASMA) ANALYSIS FOR TEST ARTICLE CONCENTRATIONS

Does the Sponsor want blood (plasma) analysis?

_____ Yes** X No

Blood (plasma) analysis will be performed by:

_____ SITEK Research Laboratories _____ Sponsor

What blood (plasma) analysis will be performed?

From the Range Finding Test?

_____ Yes _____ No

From the Assay?

_____ Yes _____ No

Test Animals: Males only.

Time points (bleeding time after the dose administration), dose levels and number of animals for the blood sample collection (unless otherwise specified by the Sponsor).

From the Range Finding Test (if sufficient pharmacokinetic information is not available), blood samples will be taken after dose administration at 3 time points (to be determined by the Sponsor), at 3 dose levels starting from the highest dose, then every other dose level. One male per dose per time point and a total of 9 males will be used.

** Additional charges will apply. See Special Services price schedule.



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From the Micronucleus Assay blood samples will be taken from all three dose levels. Only 1 time point will be used based on the plasma analysis results from the Range Finding Test or on the pharmacokinetic information of the test article (to be determined by the Sponsor). Three males per dose per time point and a total of 9 males will be used.

If requested by the Sponsor, SITEK Research Laboratories will determine the test article concentration in the plasma. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

9.0 STUDY DATES

* 9.1 Proposed Experimental Start Date: March 5, 2008

Defined as the date the animals are dosed for the Range Finding Test or Micronucleus Assay.

* 9.2 Proposed Experimental Completion Date: April 19, 2008

Defined as the last date on which data are collected directly from the study.

* 9.3 Proposed Draft Report Date: May 24, 2008

9.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

10.0 PROTOCOL APPROVAL

* <u>[Signature]</u> Study Director	<u>1-23-08</u> Date
* <u>[Signature]</u> Sponsor's Study Coordinator	<u>1-15-08</u> Date
* <u>[Signature]</u> Quality Assurance Manager	<u>1-23-08</u> Date
* <u>[Signature]</u> Safety Officer	<u>1-23-08</u> Date

* To be completed by the Testing Facility.

**SITEK RESEARCH LABORATORIES****STUDY DESIGN****PART TWO****11.0 PURPOSE**

The purpose of this study is to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes in mouse bone marrow cells (4,5).

12.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (6, 7, 8).

13.0 ABBREVIATIONS

CP	-	Cyclophosphamide
DMSO	-	Dimethyl Sulfoxide
FBS	-	Fetal Bovine Serum
IACUC	-	Institutional Animal Care and Use Committee
IP	-	Intraperitoneal
MPCE	-	Micronucleated Polychromatic Erythrocytes
MTD	-	Maximum Tolerated Dose
NCE	-	Normochromatic Erythrocytes
PCE	-	Polychromatic Erythrocytes

14.0 ANIMALS**14.1 Protocol Approval**

This protocol has been approved for the necessity of using laboratory animals by SITEK's Institutional Animal Care and Use Committee (IACUC) on February 24, 2006 (No. 06-02). In this regard, the number of animals used, the objectives of the study, the housing procedures, the rationale for animal use, the procedures involving animals, the potential for distress or pain for the animals, and the methods of euthanasia have been reviewed and approved.



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14.2 Source

Forty-two-day-old (approximately), male and female CD-1 mice will be obtained from Harlan Sprague Dawley Inc. or other acceptable vendor which routinely monitor the animals for bacterial, viral and other murine infections. The animal body weight range will be approximately 16-38 grams on delivery.

14.3 Housing and Quarantine

When the animals arrive, they will be housed according to sex in clean cages of sufficient size to allow free movement. They will be placed, one sex at a time, up to ten animals per cage, into polycarbonate cages. Hardwood chip bedding free of injurious substances will be used. The animals will receive Purina Certified Rodent Diet and fresh tap water ad libitum. The levels of contaminants present in the food and water are very low and well within acceptable levels, and are not expected to affect the outcome of the study. Bedding will be changed at least twice a week.

The animals will be quarantined for at least 7 days prior to dose administration. The animals will be observed each day, and all observations and the temperature and humidity of the animal room will be recorded in the study notebook. The animal room will be maintained at 18-26 °C and 30-70% humidity. Occasionally, minor deviations may occur due to change of outside weather conditions. A 12-hour diurnal light cycle will be employed. Only animals that are certified as healthy will be used in the study.

15.0 TEST SYSTEM IDENTIFICATION

All animals to be treated will receive an ear tag with a number unique to the particular study. All cages will be assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director, and the animal study proposal number approved by SITEK's IACUC. The microslides will be labeled with the study number, the last one, two, three or four digits of animal number as code number and date of slide preparation.

16.0 CONTROL SUBSTANCES

16.1 Positive Controls

Cyclophosphamide (CP), at 80 mg/kg (8.0 mg/mL X 10 mL/kg) as a single dose by oral gavage, will be dissolved in water and used as the positive control. The source and storage conditions for CP are given below:

Source: Sigma Chemical Company Storage Conditions: 1-5°C CAS No.: 6055-19-2
or other vendor

The specific source, lot number and expiration date of the CP will be documented in the report.

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If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

16.2 Vehicle Controls

The vehicle used for dissolving or suspending the test article will be used as the vehicle control. Deionized, distilled water, saline, corn oil (CAS #80001-30-7), dimethyl sulfoxide (CAS #67-68-5), methyl cellulose (CAS 9004-67-5), hydroxypropylmethyl cellulose (CAS #9004-65-3), tween 80 (CAS #9005-65-6) and ethanol (CAS #64-17-5) are some of the vehicles, which are compatible with this test system. If these vehicles are not suitable, others may be used with the approval of the Sponsor. The source, lot number and storage conditions of the vehicle control will be documented in the report.

17.0 DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study will be entered in a study notebook. The study notebook will also include the protocol, protocol amendments and deviations, copy of the study report, and all relevant communications with the Sponsor.

18.0 EXPERIMENTAL PROCEDURE**18.1 Determination of Solubility/Miscibility**

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test will be performed.

The test article will be tested for its solubility/miscibility in deionized, distilled water, saline, DMSO, corn oil, ethanol and/or other appropriate vehicles. Solid and viscous liquid test articles will be tested for solubility in weight per volume, and nonviscous liquids will be tested for miscibility in volume per volume or weight per volume. The solubility/miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test will consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of vehicle has been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of vehicle required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test will be conducted. 1.5 mL of vehicle in 0.1 mL increments will be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in 0.5 mL increments until 5.0 mL has been added. The resulting solution will be thoroughly mixed

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and observed for miscibility. The test article will be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations will be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article will be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data are available, the solubility/miscibility test will not be performed.

18.2 Randomization

The animals will be randomized one sex at a time and placed into experimental groups as follows: In the Range Finding Test, the animals will be randomly assigned to experimental groups, without regard to body weight, prior to dose administration (three animals per group). In the Micronucleus Assay, the animals will be weighed and placed in weight groups of 1-gram differences. Using a computer generated random number matrix, the animals will be assigned sequentially, from low to high weight groups, to cages corresponding to treatment groups (five animals per group).

18.3 Preparation of Test Article

The stock solutions for the Range Finding Test and the Micronucleus Assay will be prepared as specified in the dilution scheme which will be kept in the study notebook. The highest stock solution will be prepared by mixing together the required weight/volume of the test article and the appropriate amount of the vehicle until complete solubilization or a homogeneous suspension has been achieved. The remaining doses specified in the dilution scheme will be prepared as in the case of the highest stock solution, by performing a subsequent dilution or by varying the volume administered from the highest stock concentration to the animals. When preparing the top dosing stock and any subsequent dosing stock with a viscous or non-viscous liquid, the test article should never be diluted more than 10-fold. The dosing solutions will be prepared by SITEK study personnel just prior to treatment and kept at room temperature. In all treatments, the amount of vehicle administered to the animals will be limited to a level which has no significant toxic effect. If necessary, the test article may be administered at full strength.

18.4 Range Finding Test

The dose levels for the Micronucleus Assay will be selected according to the toxicity of the test article and in consultation with the Sponsor. If sufficient information is not available on the toxicity of the test article, a Range Finding Test will be performed. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. Unless otherwise specified by the Sponsor, the doses for the Range Finding Test are 2000, 1000, 500, 100, 50 and 10 mg/kg for solid and viscous test articles and 2.0, 1.0, 0.5, 0.1, 0.05 and 0.01 mL/kg for liquid test articles.

Treatment groups of three animals per sex will be dosed with the test article dosing solutions using the route, frequency and volume of administration selected in Section 5.0. One treatment group will be treated with only the vehicle. Food and water will be provided ad libitum. The animals will be carefully observed for 3 days after the single or the first administration of the test article. Daily records of all clinical observations and number of deaths (if any) will be kept for the treatment groups. Body weights will be checked on the



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day of dosing (Day 1) and by the end of the 3-day observation period (Day 4). All animals will be euthanized at the end of the observation period. The body weights will be entered into a computer, using MS Excel 97 Spreadsheet program. The clinical signs and mortality will be entered into a computer, using MS Word 97.

In addition to the animals treated with the test article for the standard Range Finding Test, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Range Finding Test.

The animals will be euthanized by CO₂ asphyxiation. Blood will be taken by cardiac puncture.

Approximately 0.4-1.0 mL samples of blood will be drawn into a syringe containing heparin (10,000 units). The needles will be removed from the syringes, and the blood will be transferred to an appropriately labeled 1.5 mL microfuge tubes. The tubes will be capped and placed on wet ice. Labeling will include Study number, test article identification, dose level, animal number and sampling time.

Within 0.5 hour of collection, the samples will be centrifuged at a speed of 14,000 rpm for 2 minutes. Being careful not to transfer any of the red blood cell fractions, the plasma fractions will be transferred by pipet into 1.5 mL storage vials equipped with O-ring screw caps. The samples will be stored immediately at -10 to -20°C. The frozen plasma samples will be shipped overnight on dry ice.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma in order to pick the time point for the Micronucleus Assay.

The toxicity of the test article will be evaluated on the basis of a combination of factors, namely, number of deaths, loss of body weight, and other clinical symptoms observed during the 3-day period. If possible, the highest dose selected for the Micronucleus Assay will be the Maximum Tolerated Dose (MTD) at which no deaths are recorded but animals show evidence of toxicity and/or more than 10% loss of body weight. If no toxicity is observed, the maximum dose treated will be 2000 mg/kg or 2.0 mL/kg. In addition, two lower doses, preferably one-half and one-fourth of the high dose, will be included in the assay.

18.5 Micronucleus Assay

The animals will be randomized and placed into treatment groups of five males and/or five females. One or three treatment groups will be assigned to each dose level and vehicle control. The dose levels will be as determined by the Range Finding Test after consultation with the Sponsor. One treatment group will be assigned to the positive control.

Food and water will be provided ad libitum. The test article will be administered as indicated in Section 5.0. Appropriate vehicle and positive controls will be maintained.

The animals will be euthanized by CO₂ asphyxiation. One treatment group from each test article dose level and vehicle control will be euthanized approximately 24 and 48 hours or 24, 48 and 72 hours after dose administration. Only one euthanasia, 24 hours after the


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dose administration, will be made of the animals dosed with the positive control. If multiple administrations and single harvest are selected, the animals will be euthanized approximately 24 hours after the last dose administration.

The number of animals to be used in the standard Micronucleus Assay will be as follows (if both sexes are used):

If single administration and multiple harvests are used:

For three harvests:

<u>Dose Level</u>	No. of Animals Per Harvest Time						
	24 Hours		48 Hours		72 Hours		<u>Total</u>
	<u>Males</u>	<u>Females</u>	<u>Males</u>	<u>Females</u>	<u>Males</u>	<u>Females</u>	
High	5	5	5	5	5	5	30
Mid	5	5	5	5	5	5	30
Low	5	5	5	5	5	5	30
Vehicle Control	5	5	5	5	5	5	30
Positive Control	5	5	N/A	N/A	N/A	N/A	<u>10</u>
							130

For two harvests:

<u>Dose Level</u>	No. of Animals Per Harvest Time				
	24 Hours		48 Hours		<u>Total</u>
	<u>Males</u>	<u>Females</u>	<u>Males</u>	<u>Females</u>	
High	5	5	5	5	20
Mid	5	5	5	5	20
Low	5	5	5	5	20
Vehicle Control	5	5	5	5	20
Positive Control	5	5	N/A	N/A	<u>10</u>
					90

If multiple administrations and single harvest are used:

<u>Dose Level</u>	No. of Animals Per Group		
	<u>Males</u>	<u>Females</u>	<u>Total</u>
High	5	5	10
Mid	5	5	10
Low	5	5	10
Vehicle Control	5	5	10
Positive Control	5	5	<u>10</u>
			50

After the animal has been euthanized, the groin area will be cleansed with 70% ethanol, and both femurs will be exposed by cutting into the skin and muscle of the thighs. The femurs will be separated just above the kneecaps, and the femur heads will be removed



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with scissors. The bone marrow from both femurs will be flushed into a medium disposable culture tube (13x100 mm), containing 1.0 mL of FBS, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes will be centrifuged at 800 rpm for 5 minutes, and the supernatant will be removed, leaving approximately 0.1 mL of serum above the cell pellet. The cell pellet will be resuspended in the remaining serum until a homogeneous suspension is observed.

A small drop of the cell suspension will be placed on the unfrosted end of a clean microslide and spread along the length of the slide. The slides will be allowed to air dry, fixed in methanol for 15 minutes, and allowed to air dry again. The slides will be stained in Wright-Giemsa stain for 2-4 minutes, rinsed in distilled water, allowed to air dry completely, and mounted in Permount using #1 cover glasses. The backs of the slides will be cleaned with methanol.

The slides will be scored "blind" in order to avoid bias on the part of the scorer(s). The number of PCE among total erythrocytes (PCE + NCE) will be determined for each animal by counting a total of at least 200 erythrocytes. The number of MPCE then will be scored for 2000 PCE per animal (2).

In addition to the animals treated with the test article for the standard Micronucleus Assay, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Micronucleus Assay.

The animals will be euthanized by CO₂ asphyxiation. Blood will be taken by cardiac puncture.

The procedures for blood collection, storage and shipping will be the same as in the Range Finding Test.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma. There is sufficient correlation for measurements of test article in plasma to be adequate for validating bone marrow exposure (3).

18.6 Statistical Analysis

The data will be analyzed separately for male and female animals. The data from the score sheets will be consolidated into summary sheets and entered into a computer using a MS Excel 97 validated spreadsheet program. The treatment group means will be calculated for the percentage of PCE among total erythrocytes, as well as the frequency of MPCE. A significant reduction (more than 20% versus that of the control vehicle) will be used as an indication of toxicity. Unless otherwise indicated, the frequency of MPCE in each treatment group will be compared to that in the respective vehicle control using a one-tailed Student's t-test (9). Epistat statistical package will be used to calculate p values for the t-test. The results will be considered significant if the p value is ≤ 0.025 . The Cochran-Armitage Test (trend test) (10) will be used for evidence of a dose-related response, if the Student's t-test shows a positive result. The trend test will be considered significant if the p value is ≤ 0.05 .

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Historical vehicle control values also will be taken into consideration when necessary as described in Section 18.8.1. Statistical analysis will not be performed if the test dose value is equal to or less than the concurrent or historical vehicle control.

18.7 Criteria for a Valid Assay

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed ten.
2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.
3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

18.8 Evaluation of Test Results**18.8.1 Positive Response**

The test article will be considered to have caused a positive response in this assay if:

1. The test article shows a positive dose-response trend and a statistically significant increase ($p \leq 0.025$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article causes a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may be compared to historical vehicle control data.
2. In the event there is no positive dose-response trend, at least two consecutive test doses show a statistically significant increase in the number of MPCE.

18.8.2 Negative Response

The test article will be considered to have caused a negative response if none of the test doses show a statistically significant increase in the number of MPCE when compared to the vehicle control.

18.8.3 Equivocal Response

If the test article induces a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend, the results will be considered equivocal. In such a case, a repeat assay will be performed with the approval of the Sponsor.

**SITEK RESEARCH LABORATORIES****18.8.4 Other Considerations**

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

19.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor, and these changes and the reasons for them will be documented. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and these changes, along with the reasons for them or explanations, will be documented. If the amendments or deviations involve animal use, they will be reviewed by the IACUC. Protocol amendments and deviations will be appended to the protocol.

20.0 REPORT OF RESULTS**20.1 Content**

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study, and the dates on which the study was initiated and completed.
2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
3. Statistical methods employed for analyzing the data.
4. The test and control articles identified by name, chemical abstracts number or code number, strength, purity and composition, or other appropriate characteristics.
5. A description of the methods used.
6. The name, sex and source of the animals used.
7. A description of the treatment procedures, vehicle used for treatment and duration of treatment.
8. A description of all circumstances that may have affected the quality or integrity of the data.



SITEK RESEARCH LABORATORIES

9. The name of the Study Director and the names of other technical personnel or other professionals, who participated in performing the study.

10. A description of the transformations, calculations or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

11. The signed and dated final report of the Study Director and Quality Assurance Manager.

12. The location where the raw data and final report are to be stored.

13. A statement from the Quality Assurance Unit.

20.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments and will include the reasons for the changes. Report amendments will be added to the final report as an addendum.

21.0 ARCHIVES

The raw data, documentation, protocol and Final Report, along with an electronic file containing the data tables and copy of the Final Report of the study will be maintained in SITEK Research Laboratories' Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, for ten years. All raw data, documentation, and the final report of all Subcontractors/Sponsor work will be maintained by the Subcontractor/Sponsor.

22.0 REFERENCES

1. United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

2. OECD Guideline for the Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

4. Heddle, J. A. A rapid in vivo test for chromosomal damage. *Mutat. Res.*, **18**, 187-190, 1973.

5. Schmid, W. The micronucleus test. *Mutat. Res.*, **31**, 9-15, 1975.



SITEK RESEARCH LABORATORIES

6. Mavournin, K. H., et al. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.*, 239, 29-80, 1990.
7. Hayashi, M., et al. In vivo rodent erythrocyte micronucleus assay. *Mutat. Res.*, 312, 293-304, 1994.
8. Tice, R. R. and M. D. Shelby. Report of in vivo subgroup. *Mutat. Res.*, 312, 287-292, 1994.
9. Lovell, D. P., et al. Statistical analysis of in vivo cytogenetic assays in: D. J. Kirkland (Ed.) *Statistical Evaluation of Mutagenicity Test Data*. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge. pp. 184-232, 1989.
10. Margolin, B. H., et al. Statistical analysis for the in vitro cytogenetic assay using Chinese hamster ovary cells. *Enviro. Mutagen.*, 8, 183-204, 1986.

PROTOCOL AMENDMENT

Amendment No.: 1

Sponsor: USA RDECOM, AMSRD-MSF, Environmental
Acquisition & Logistics Sustaining Program
Aberdeen Proving Ground, MD 21010

Testing Facility: SITEK Research Laboratories
15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

SITEK's Study No.: 0976-1521

Sponsor's Study No.: N/A

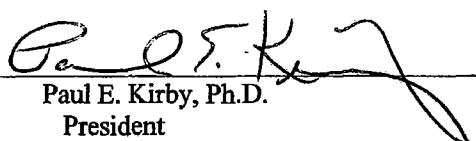
Test Article I.D.: Dimethylamine-2-ethyl azide (DMAZ)

Protocol Title: *In Vivo* Test for Chemical Induction of
Micronucleated Polychromatic Erythrocytes in
Mouse Bone Marrow Cells

Amendment No. 1: Protocol Page 1, Section 2.3. The study director
has been changed from Dr. Shambhu K. Roy to
Dr. Jian Song.

Reason for Amendment No. 1: Dr. Shambhu K. Roy is no longer employed by
SITEK Research Laboratories.

APPROVED:



Paul E. Kirby, Ph.D.
President

6-25-08
Date

PROTOCOL AMENDMENT

Amendment No.: 2

Sponsor: USA RDECOM, AMSRD-MSF
Environmental Acquisition & Logistics
Sustaining Program
Aberdeen Proving Ground, MD 21010

Testing Facility: SITEK Research Laboratories
15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

SITEK's Study No.: 0976-1521

Sponsor's Study No.: N/A

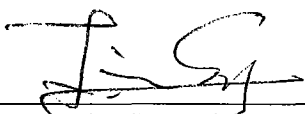
Test Article ID: DMAZ

Protocol Title: In Vivo Test for Chemical Induction of
Micronucleated Polychromatic Erythrocytes in Mouse
Bone Marrow Cells

Amendment No. 2: Protocol page 11, Section 18.4. Based on the results of
the Range Finding Test, the dose levels for Micronucleus
Assay will be 500, 250 and 125 mg/kg.

Reason for Amendment No. 2: Protocol Page 11, Section 18.4. The actual dose levels for
the assay, once determined, will be added to the protocol
in the form of an amendment.

APPROVED:



Jian Song, Ph.D.
Study Directory

4-29-08
Date

PROTOCOL DEVIATION

Deviation No.: 1

Sponsor: USA RDECOM, AMSRD-MSF
Environmental Acquisition & Logistics
Sustaining Program
Aberdeen Proving Ground, MD 21010

Testing Facility: SITEK Research Laboratories
15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

SITEK's Study No.: 0976-1521

Sponsor's Study No.: N/A


Test Article ID: DMAZ

Protocol Title: In Vivo Test for Chemical Induction of
Micronucleated Polychromatic Erythrocytes in Mouse
Bone Marrow Cells


Deviation No. 1: Protocol Page 9, Section 14.3 Housing and Quarantine:
As per protocol, the animal room should have been
maintained at 18-26 °C and 30-70% humidity. However
the temperature range in this study was 17-23 °C and the
humidity range was 17-54%.

Reason for Deviation No. 1: This deviation occurred due to changes in outside
weather conditions. However, this deviation does not
significantly affect the outcome of the study.

APPROVED:



Jian Song, Ph.D.
Study Director



Date

PROTOCOL DEVIATION

Deviation No.:	2, 3 and 4
Sponsor:	USA RDECOM, AMSRD-MSF Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0976-1521
Sponsor's Study No.:	N/A
Test Article ID:	DMAZ
Protocol Title:	In Vivo Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells
<u>Deviation No. 2:</u>	Protocol Page 11, Section 18.4: The body weights will be entered into a computer, using MS Excel 97 Spreadsheet program. The clinical signs and mortality will be entered into a computer, using MS word 97. However, MS excel 2003 and MS word 2003 were used in the test.
<u>Reason for Deviation No. 2:</u>	The computer program was upgraded.
<u>Deviation No. 3:</u>	Protocol Page 12, Section 18.5: One or three treatment groups will be assigned to each dose level and vehicle control. However, two groups were assigned to each dose level and vehicle control.
<u>Reason for Deviation No. 3:</u>	Two groups (male and female) were assigned to each dose level and vehicle control.
<u>Deviation No. 4:</u>	Protocol Page 14, Section 18.5: The slides will be stained in Wright-Giemsa stain for 2-4 minutes, rinsed in distilled water, allowed to air dry completely and mounted in Permout using #1 cover glasses. However

Cytoseal was used to mount the slides in the test.

Reason for Deviation No. 4:

Cytoseal was used in the lab recently.

APPROVED:



Jian Song, Ph.D.
Study Director

5-2-08

Date

APPENDIX IV

CERTIFICATE OF ANALYSIS

JAN-15-2008 14:46 From:USACHPPM TOX
Jan 15, 2008 11:56AM

4104368258

To:3019268891

P.3/9

V: 1855 F 2

**MACH I®****Certificate of Analysis****3M DMAZ L-15686****LOT 8****Orbital Technologies**

Parameter	Results
Water	0.10%
Specific Gravity	0.928
Refractive Index	1.440
Flash Point	88° F
pH	9.2
% purity by gas chromatography	99.3 area %
Date of Manufacture/Testing	December 2002

Note: It is recommended that this product be kept in refrigeration for long term storage.

Signed by: B. Decker 10/4/07
MACH I Inc. Representative

MACH I Inc., 340 East Church Road, King of Prussia, PA 19046
Phone: (610) 279-2340 Fax: (610) 279-8505 website www.machichemicals.com

JAN-15-2008 11:56

From: 610 279 6644

ID:USACHPPM TOX